

Article

Compensatory Proliferation in *Drosophila* Imaginal Discs Requires Dronc-Dependent p53 Activity

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Summary

Background: The p53 transcription factor directs a transcriptional program that determines whether a cell lives or dies after DNA damage. Animal survival after extensive cellular damage often requires that lost tissue be replaced through compensatory growth or regeneration. In *Drosophila*, damaged imaginal disc cells can induce the proliferation of neighboring viable cells, but how this is controlled is not clear. Here we provide evidence that *Drosophila* p53 (dp53) has a previously unidentified role in coordinating the compensatory growth response to tissue damage.

Results: We find that dp53, the sole p53 ortholog in *Drosophila*, is required for each component of the response to cellular damage, including two separate cell-cycle arrests, changes in patterning gene expression, cell proliferation, and growth. We demonstrate that these processes are regulated by dp53 in a manner that is independent of DNA-damage sensing but that requires the initiator caspase Dronc. Our results indicate that once induced, dp53 amplifies and sustains the response through a positive feedback loop with Dronc and the apoptosis-inducing factors Hid and Reaper.

Conclusions: How cell death and cell proliferation are coordinated during development and after stress is a fundamental question that is critical for an understanding of growth regulation. Our data suggest that dp53 may carry out an ancestral function that promotes animal survival through the coordination of responses leading to compensatory growth after tissue damage.

Introduction

Much of the cellular response to DNA damage and other cellular stresses is coordinated by the p53 transcription factor [1]. In irradiated mammalian cells, p53 is stabilized and activated in response to signaling from the damage-sensing kinase ATM, ataxia telangiectasia mutated [2]. Upon activation by ATM and the downstream kinase Chk2, p53 directs a transcriptional program that results in G1 or G2 cell-cycle arrest, which facilitates repair of genomic damage, or in apoptosis [3]. By inducing these processes, p53 ensures the safety of the genome and thus also of the animal. However, survival after extensive cellular damage often requires that lost tissue be replaced through compensatory growth or regeneration. How compensatory growth is initiated in response

to tissue damage is not known, but experiments in several systems have suggested that dying cells contribute by emitting autocrine and paracrine survival and growth-stimulating signals [4–9]. In *Drosophila* imaginal discs, the epithelial primordia for adult structures, compensatory growth is induced by many insults, including irradiation, disc fragmentation, and genetic manipulation [10–12]. Recent work indicates that compensatory proliferation is also induced in imaginal discs containing “undead” cells, damaged by irradiation or ectopic expression of proapoptotic genes but protected from death by the baculovirus caspase inhibitor P35 [5–7]. Under these conditions, the undead cells remain in the epithelium despite receiving a constant death signal; as a result, the discs undergo prolonged growth and express the pattern-organizing genes *wingless* (*wg*) and *decapentaplegic* (*dpp*) ectopically. *Wg* and *Dpp* have been implicated in the signaling events that accompany compensatory proliferation induced by undead cells and also in regeneration [7, 13–17]. In addition, it is thought that the initiator caspase Dronc, a homolog of caspase-9, has a nonapoptotic role in the undead-induced compensatory growth [5].

Drosophila has one p53 ortholog, *dp53*, which is necessary for apoptosis after irradiation [18–23]. To date, *dp53* has not been shown to have a role in damage-induced growth arrest [18–23]. However, homozygous *dp53* mutants are hypersensitive to ionizing irradiation, with reduced viability at late larval and pupal stages [20, 23]. This raises the interesting possibility that tissue recovery after cellular damage is impaired in *dp53* mutant animals. p53 is not necessary for normal development in vertebrates or in flies, although its loss in mice can lead to spontaneous tumor formation due to accumulation of damaged cells [3]. In contrast, the vertebrate p53 paralogs, p63 and p73, which do not exist in *Drosophila*, are required for specific developmental processes unrelated to DNA damage. For example, mice lacking p63 have severe epidermal and limb abnormalities, arising in part from an epidermal stratification failure and inadequate renewal of basal progenitor cells [24–26].

The role of p53 as director of many aspects of the DNA-damage response puts it in a position to coordinate additional processes related to tissue damage. This prospect, along with the roles of its close relative p63 and the reduced survival of irradiated *dp53* mutants, prompted us to ask whether *dp53* plays a regulatory role in the growth response after cellular damage in *Drosophila*. We have used the generation of undead cells to induce cellular damage and compensatory proliferation and examined this process in detail. We report here that prior to the onset of growth, a disc-wide response is generated, with many undead cells transiently arresting cell division while other, viable cells in the disc arrest permanently. Compensatory proliferation then occurs with normal kinetics, but results in prolonged larval development. We find that the cell-cycle arrests, the subsequent

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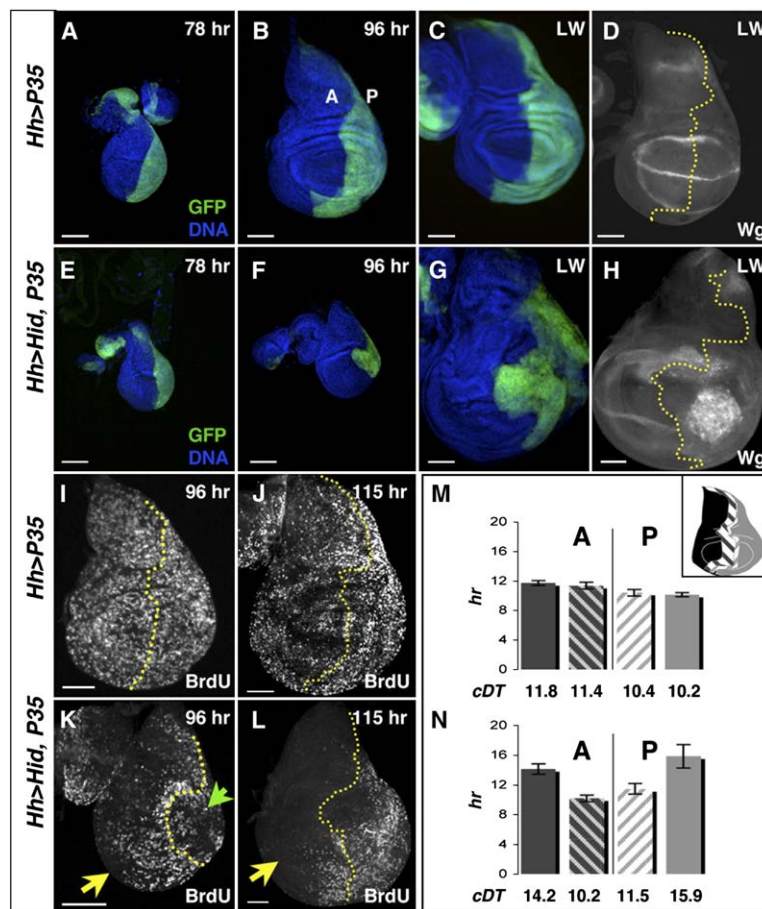


Figure 1. Undergrowth Is the First Detectable Phenotype of Undead Cells

(A–D) Control wing imaginal discs expressing UAS-P35 in posterior cells (green) under Hedgehog (Hh) Gal4 control. In this and all subsequent images, anterior is left, dorsal is up. A, anterior; P, posterior.

(A) 78 hr disc.

(B) 96 hr wing imaginal disc.

(C) Late wandering stage (LW) wing disc, near the end of the disc growth period.

(D) LW wing disc stained for Wingless (Wg) protein.

(E–H) Wing discs with undead cells in the posterior.

(E) 78 hr wing disc, similar in size to the control (A).

(F) By 96 hr, the wing discs with undead cells are smaller than controls (compare with [B]), with a small, distorted posterior compartment (green).

(G) LW stage wing disc with undead cells in the posterior. This disc is 158 hr old and substantially overgrown in both the anterior (blue) and the posterior (green) compartments. Only the posterior compartment contains undead cells.

(H) LW wing disc with undead posterior cells, stained for Wg protein. Note that Wg is expressed ectopically; these Wg-expressing cells clump together near the center of the posterior compartment.

(I and J) BrdU incorporation in 96 hr (I) and 115 hr (J) control wing discs.

(K) BrdU incorporation in the 96 hr disc from (F). Note the lack of BrdU incorporation in the posterior undead cells (green arrowhead) and in the anterior (yellow arrow). Cells along the anterior-posterior (A/P) boundary (marked by dotted yellow line) continue to incorporate BrdU.

(L) 115 hr wing disc with undead cells, showing that undead cells have begun to incorporate BrdU again, along with the anterior cells along the A/P boundary. However, lateral anterior cells remain quiescent (yellow arrow). In all images, scale bars show relative size.

(M) Cell proliferation rates in control wing discs. Gray bars, posterior cells; black bars, anterior cells. Each bar represents a region of the wing disc as depicted in the inset: solid bars, lateral regions; scored bars, medial regions. Cell doubling times (cDT), below each bar, of lateral cells and medial cells are similar; posterior cells cycle moderately faster than anterior cells.

(N) Cell proliferation rates in wing discs with posterior undead cells. Undead, posterior, lateral cells cycle significantly slower than controls ($p < 0.003$). Anterior lateral cells also cycle slower than control anterior lateral cells ($p < 0.002$). The anterior and posterior lateral proliferation rates are likely to be an underestimate, given that BrdU incorporation was normal for the first 8–10 hr of the 26 hr clonal growth period. In contrast, cells in medial regions of both compartments cycle with normal kinetics.

Error bars in (M) and (N) denote standard errors of the mean.

compensatory growth, and the patterning gene alterations induced by undead cells all require *dp53*. Strikingly, *dp53* activity under these conditions is not dependent upon DNA-damage sensing; rather, it requires a nonapoptotic role of the Dronc caspase. Our data indicate that *dp53* activity is at the center of a feedback loop involving Dronc and the proapoptotic genes *hid* and *reaper* that amplifies and sustains compensatory proliferation. Finally, we demonstrate that in a model of disc regeneration, the formation of a blastema, a step necessary for compensatory proliferation during regeneration, is impaired in *dronc* and *dp53* mutants.

Results

To create undead cells, we used the Gal4/UAS system to coexpress the UAS-regulated prodeath genes *Hid* or *Reaper* (Rpr) and the caspase inhibitor UAS-P35 in

posterior cells, with the Hedgehog Gal4 (HhGal4) driver. In all of our experiments, the undead cells were marked by coexpression of GFP. As reported previously, expression of *Hid* or *Rpr* alone was lethal at early larval stages, but expression of either proapoptotic gene with P35 allowed survival until early pupal stages (data not shown) [5, 7]. The development of *Hid* or *Rpr* + P35-expressing animals was significantly delayed. Embryogenesis and the first two larval instars progressed with appropriate timing, but the last instar continued 3–4 days longer than controls (see Figure S1A in the Supplemental Data available with this article online). Early in the last instar (78 hr after egg laying [AEL]), wing discs containing undead cells were similar in size and morphology to 78 hr control discs (Figures 1A and 1E). However, by 96 hr AEL, the discs were significantly smaller than controls, suggesting that their growth was blocked. The posterior compartments, containing

undead cells, were disproportionately small and distorted (Figures 1B and 1F; Figure S1B). However, within the next 24 hr the discs began to grow again, eventually losing their normal monolayered architecture and taking on extra folds (Figure 1G). By the end of the prolonged growth period, the discs were up to 48% larger than control discs (Figure S1B). As observed previously, expression of *Hid* or *Rpr* + *P35* led to ectopic expression of the pattern regulators *wingless* (*wg*) and *decapentaplegic* (*dpp*), beginning between 87 and 96 hr AEL. Their expression was restricted to undead cells and located near the endogenous *wg* and *dpp* expression domains (Figures 1D and 1H and data not shown) [5–7]. Undead cells created with expression of *Rpr* + *P35* gave similar phenotypes, as did the use of another posterior-specific Gal4 driver, *Engrailed* Gal4 (Figures S1A and S1B).

Cell-Cycle Arrests Precede Compensatory Proliferation

We investigated the reason for the small disc size at 96 hr AEL by labeling cells with the DNA replication marker BrdU. At 78 hr AEL, when still the same size as controls, wing discs with undead cells incorporated BrdU with the same general pattern as 78 hr control discs (data not shown). By 96 hr, most undead cells no longer incorporated BrdU, and remarkably, anterior cells far from the undead cells also proliferated sparsely (Figure 1K). By contrast, cells flanking the A/P boundary in both compartments continued to incorporate BrdU.

To examine the alterations in cell proliferation in more detail, we measured proliferation rates during the time disc growth was slowed. We used flip-out clonal analysis [27, 28] to generate cell clones marked by expression of β -galactosidase randomly throughout the disc. This assay estimates the rate of cell proliferation (cell division + cell survival) that occurs in the wing disc over a defined period of time. Clones were induced while cells still proliferated normally, at 70 hr AEL, and allowed to grow until 96 hr AEL. At the end of this growth period, we counted the number of β -gal-positive cells that had accumulated in each clone to determine the rate of their proliferation. Since we had observed proliferation differences across the disc, the location of each clone in the disc was also noted (Figures 1M and 1N, inset). By these measurements, posterior cells located laterally (with respect to the A/P boundary; see inset) doubled every 10.2 hr in control discs, but when the cells expressed *Hid* + *P35*, they took 15.9 hr to double, nearly 6 hr longer (Figures 1M and 1N; $p < 0.003$). Likewise, anterior cells located laterally proliferated significantly slower than comparable control cells (14.2 hr versus 11.8 hr, respectively, $p < 0.002$; Figure 1M versus 1N). All the cells in the disc proliferated normally prior to 87 hr AEL, so these proliferation rates are likely to be an underestimate. In contrast to the slow-proliferating cells, the BrdU-incorporating cells on both sides of the A/P border divided at the same rate as controls (Figures 1M and 1N).

To determine which phases of the cell cycle were affected in the slow-growing cells, we analyzed wing disc cells via flow cytometry at three time points spanning the third instar, using GFP expression to identify the posterior, undead cells. In control wing discs, cell cycles shifted from G1 to G2 regulation between 96 hr and late wandering (LW) stage (Figure 2A) [28, 29]. In

contrast, at 96 hr, undead cells had accumulated in G2 and were 70% larger than anterior cells (Figure 2B). Expression of the *Cdc25* homolog *string* (*stg*) mRNA, which is rate limiting for progression into mitosis and tightly transcriptionally controlled, was reduced or absent from many undead cells, indicating that cells had arrested in G2 (Figure 2E [$n = 36$]) [30, 31]. Their large size suggested that although blocked in G2, the undead cells continued to grow in size. Later, at early wandering (EW) stage, undead cells re-entered the cell cycle, expressed *stg* again, and by LW proliferated with a G1-regulated cell cycle more appropriate of younger cells (Figures 2A, 2B, and 2F). Consistent with this, undead cell size decreased during this period and by LW was similar to controls (Figure 2B, LW).

In contrast to the posterior, undead cells, the cell-cycle profile of anterior cells was similar to controls at 96 hr (Figure 2B versus 2A). However, we suspect that this reflected the large population of proliferating cells at the A/P boundary, since many cells at the lateral anterior edge of the wing discs lacked *stg* mRNA, suggesting that, like the undead cells at this stage, they had arrested in G2 (Figures 2E [$n = 36$] and 2F [$n = 20$]). This anterior cell arrest was permanent, because *stg* (and BrdU) was absent from most of the cells at all stages after 96 hr.

These data indicate that the earliest detectable consequence of tissue damage due to undead cells is a G2 arrest in two separate cell populations, which explains the small wing disc size at 96 hr of development. Our results indicate that tissue damage imposed by undead cells leads to global signaling that instructs cells to arrest either transiently or permanently or to proliferate. Interestingly, many undead cells were capable of proliferating after their initial arrest and apparently contributed to the eventual disc overgrowth since most of these cells incorporated BrdU despite a significant level of *Hid* and activated caspase-3 (Figures 3A and 3B [$n = 23$ and 27, respectively] and data not shown). However, cells with highest levels of *Hid* and caspase activity were often devoid of BrdU (Figure 3A, inset and data not shown). Unlike control wing discs, cell proliferation in discs with undead cells continued into pupal development; the cells did not differentiate and eventually disintegrated (data not shown).

dp53 Is Induced in Undead Cells and Is Required for the Growth Alterations

The G2 arrests in both cell populations were reminiscent of the behavior of irradiated imaginal discs and suggested that a damage response had been activated [10, 18, 19, 23, 32]. We examined flies carrying mutations in genes in the DNA-damage-sensing pathway, including *atm* and *chk2*. *Drosophila atm* is essential during development, and although mutant animals are very sick, they are viable until the pupal stage [33]. We examined *atm*⁶ null mutant wing discs containing undead cells and found that they expressed *wg* ectopically and overgrew relative to control *atm*⁶ mutant discs. *chk2* null mutant wing discs behaved similarly; thus, neither *atm* nor *chk2* were required for the compensatory proliferation or ectopic expression of *Wg* (Figure 3F; Figure S2). Consistent with these results, we found that very few cells in wing discs with undead cells expressed the

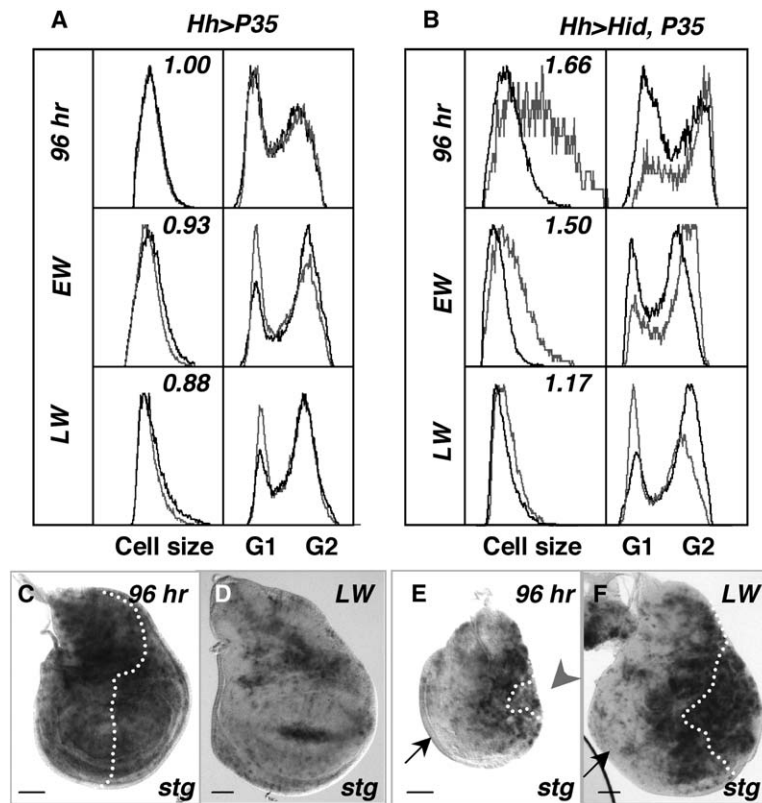


Figure 2. Autonomously and Nonautonomously G2 Arrests Precede Compensatory Proliferation

(A) Cell-cycle profiles and cell size from control wing discs at 96 hr, early wandering (EW), and late wandering (LW) stage. Posterior cells are marked GFP and are represented by gray traces; black traces represent profiles of anterior cells. Left boxes: relative cell size, measured by forward scatter. The number in these boxes is the ratio of posterior/anterior cell size. Right boxes: cell-cycle profiles.

(B) Cell-cycle profiles and cell size from wing discs with undead cells. Undead cells (posterior), wild-type cells (anterior). Trace colors are same as controls. At 96 hr, most undead cells are in G2 and nearly 1.7× larger than anterior cells. At EW stage, the undead cells are somewhat smaller, but most still remain in G2. By LW stage, undead cells are similar in size to anterior cells, but their cell cycles have shifted to one similar to younger control wing discs (96 hr and EW). At LW stage, the wild-type anterior cells (black trace) adopt a cell cycle that is predominantly G2 regulated, the characteristic profile of LW stage wing discs (e.g., control discs in [A], LW).

(C–F) *string^{cdc25}* (*stg*) mRNA expression in control wing discs at 96 hr AEL (C) and LW (D), and in discs with undead posterior cells at 96 hr AEL (E) and LW (F). Wing discs are all oriented with the posterior to the left. *stg* is expressed in a periodic fashion throughout the control wing disc at 96 hr (C), but by LW

stage most cells in control discs do not express *stg* and are arrested in G2 (D). In discs with undead cells, *stg* is expressed in anterior cells and posterior cells surrounding the A/P boundary at 96 hr but is largely missing in lateral anterior cells and in most undead cells (E). Later, when the undead cells are proliferating, *stg* is expressed at high levels in these cells; however, note that it is still absent in lateral anterior cells (F). Scale bars in (C)–(F) represent 50 μm.

DNA-damage marker γ-H2AX, suggesting that the DNA-damage response had not been activated (data not shown).

Nevertheless, *dp53* mRNA was dramatically induced, specifically in the undead cells (Figure 3C [n = 26 discs]). To test whether *dp53* was required for undead cell-induced effects, we used two different targeted null alleles of *dp53* [20, 21]. Remarkably, we found that loss of *dp53* completely prevented all of the phenotypes associated with the growth arrests caused by undead cells. In *dp53* mutants, both the undead cells and lateral anterior cells incorporated BrdU normally throughout development (Figure 3G [n = 24 discs]). Despite the persistent expression of *Hid* + *P35*, loss of *dp53* also suppressed the subsequent compensatory proliferation and the ectopic expression of *wg* in these discs (Figures 3G and 3H; Figure S2). Furthermore, *dp53* mutant larvae expressing *Hid* + *P35* developed with normal timing (Figure S1A). Thus, *dp53* activity is necessary for all of the growth and patterning alterations associated with undead cells, suggesting that it has a role in the mechanism of their development.

Dronc Is Required for *dp53* to Regulate Growth

Our results suggest that the DNA-damage sensors ATM and Chk2 are not required for *dp53* to function in wing discs containing undead cells. The AMP-activated protein kinase (AMPK) has been shown to activate *dp53* upon metabolic stress [34], and therefore we examined

levels of the active, phospho-AMPK in discs with undead cells. However, p-AMPK was present at levels similar to controls (Figures S3D and S3F).

We noticed that in spite of the complete lack of a response in *dp53* mutants, the undead cells still contained high levels of cleaved caspase-3 (Figure 3I [n = 24]). *Hid* and *Rpr* initiate the death process by preventing the inhibitor of apoptosis, Diap-1, from blocking both initiator and effector caspase (e.g., caspase-3) activation [35]. Although the caspase inhibitor *P35* prevents the activity of executioner caspases such as caspase-3, it does not prevent their processing by (for example) the *P35*-insensitive Dronc; thus, the presence of the cleaved form suggested that Dronc was still active in *dp53* mutant discs [35]. Since Dronc had previously been implicated in a nonapoptotic role in undead cell-induced compensatory proliferation [5], we examined whether it was required for *dp53* function. We generated undead cells by using *EnGal4* to drive expression of *Hid* + *P35* in animals carrying a null allele, *dronc¹²⁹* [36]. Loss of just one copy of *dronc* substantially reduced the growth alterations and allowed imaginal cells to differentiate and adults to form, suggesting that Dronc was haploinsufficient for the effects induced by undead cells (Figures S3A and S3B). Interestingly, high levels of cleaved caspase-3 were still induced in these *dronc¹²⁹* heterozygotes, which suggests that Dronc's caspase-activating function may operate at a different activity threshold than its role in compensatory proliferation (Figure S3A)

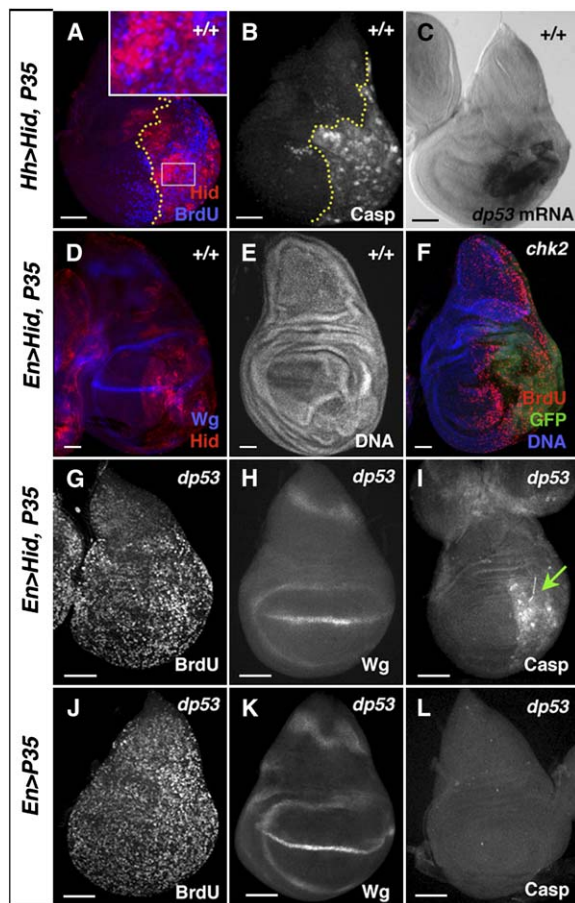


Figure 3. *dp53* Is Transcriptionally Induced in Undead Cells Independent of DNA-Damage Sensing

(A) 115 hr wing disc expressing Hid + P35 under HhGal4 control in the posterior. The A/P boundary is marked by a dotted yellow line. Undead cells express Hid protein (red) and, except for those with the highest level of Hid, also incorporate BrdU.

(B) Similar disc with undead posterior cells, stained with an antibody against the cleaved form of caspase-3, which marks most undead cells and also some anterior, wild-type cells. The A/P boundary is marked by a dotted yellow line.

(C) *dp53* mRNA is specifically upregulated in undead cells.

(D and E) Wing discs expressing Hid + P35 in posterior cells with Engrailed (En) Gal4 driver, showing ectopic Wg protein (D) and overgrowth (E).

(F) *chk2* mutant wing disc with posterior undead cells (in this genotype induced by HhGal4). Loss of *chk2* does not alleviate the overgrowth or the anterior cell-cycle arrest.

(G–L) Loss of *dp53* completely alleviates the cell-cycle arrests and undergrowth of 96 hr wing discs caused by undead cells.

(G) BrdU incorporation in a 96 hr *dp53* mutant wing disc with posterior undead cells. Neither posterior cells, expressing Hid + P35, nor anterior cells are arrested. The size of the posterior compartment and of the entire disc is normal. Despite the presence of undead cells, the discs have normal morphology.

(H) Wg protein is not ectopically expressed in these *dp53* mutant discs.

(I) Caspase-3 is still present in the cleaved form, indicating that Hid still induces the apoptotic signal (green arrow).

(J–L) Control *dp53* mutant wing discs, expressing P35 alone. Bars indicate relative scale.

[5]. In homozygous *dronc*¹²⁹ mutants with posterior undead cells, the extra growth and ectopic *wg* expression induced by undead cells were completely abolished

(Figures 4A, 4B, 4D, and 4E). Complete loss of *dronc* also abolished caspase-3 cleavage in 100% of the wing discs, even though cells expressed high levels of Hid; this suggests that Dronc was responsible for caspase-3 cleavage under these conditions (Figure 4E [n = 21]). Moreover, *dp53* mRNA was not increased in undead cells of *dronc*¹²⁹ mutant wing discs (Figure 4J [n = 34]).

Dronc activity is held in check by Diap1, which is degraded upon binding by HRG (Hid-Rpr-Grim) proteins [35]. We tested whether Diap1 degradation was required for compensatory growth by coexpressing Dronc + P35 in the discs, bypassing Diap1. Diap1 protein was lost in most undead cells expressing Hid or Rpr + P35 (Figure 4F), but persisted in many Dronc + P35-expressing undead cells (Figure 4G). Despite the presence of Diap1 protein, expression of Dronc + P35 led to more severe growth effects than did expression of Hid or Rpr + P35 (Figure 4G [n = 36 discs]). These results confirm a previous report that loss of Diap1 is not required for compensatory proliferation induced by undead cells [5]. The expression of Dronc + P35 was sufficient to induce *dp53* mRNA in wild-type wing discs (Figure 4K). However, Dronc expression was not able to induce overgrowth or loss of Diap1 in *dp53* mutant wing discs (Figure 4H [n = 20 discs]). Taken together, our experiments indicate that Dronc is both necessary and sufficient to induce *dp53* expression and activity in wing discs, leading to growth arrest and subsequently to compensatory proliferation.

***dp53* Amplifies and Sustains Signaling from Undead Cells through a Positive Feedback Loop**

Irradiation induces *dp53*-dependent expression of *rpr* and *hid* in embryos, and *rpr* is a direct target of *dp53* [18, 19]. In our experiments, expression of Hid + P35 induced expression of *rpr*, and expression of Rpr + P35 induced expression of *hid*, suggesting a feedback relationship between the two genes (Figures 5A and 5B [n = 15 and 23 discs, respectively]). Expression from the endogenous *rpr* locus required *dp53*, as shown by the fact that *dp53* mutant wing discs expressing Hid + P35 no longer expressed *rpr* (Figure 5C [n = 32]). Loss of *rpr* or *hid* had little effect on these phenotypes, consistent with the induction of both of these genes in response to undead cells (data not shown). However, feedback regulation between *dp53* and *hid* and *rpr* required *dronc*, because *dronc*¹²⁹ mutant discs that expressed exogenous Hid + P35 did not induce endogenous *rpr* expression (Figure 5D [n = 27]). Thus, a regulatory loop, consisting of the initiator caspase Dronc, *dp53*, and the endogenous *hid* and *rpr* loci, drives the disc response to undead cells. *dp53* mutants containing undead cells were unhealthy, however, and only 30% of larvae survived to wandering stage, possibly due to the presence of TUNEL-positive cells despite inhibition of effector caspase activity by P35 (Figures S3C and S3E). It is possible that in the absence of a *dp53*-driven program of growth, caspase-3-independent cell death (presumably induced by Dronc activity), contributed to the demise of the animal. This interpretation is consistent with reports of the reduced survival of irradiated *dp53* mutant animals [20, 23].

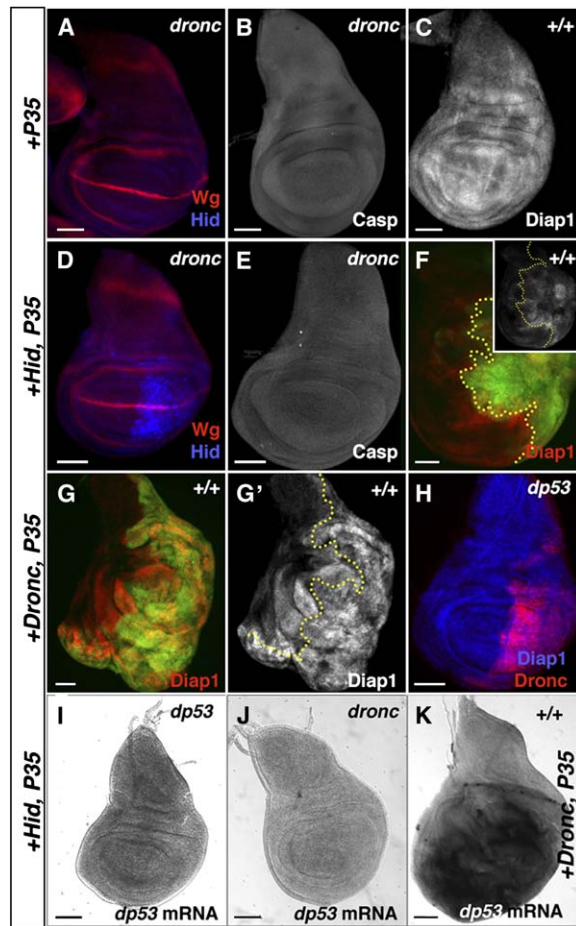


Figure 4. Dronc Activity Is Required for dp53 to Regulate Compensatory Proliferation

(A, B, D, E) Dronc is required for dp53 activation.
(A and B) Control *dronc* mutant wing discs stained for Wg (A) and cleaved caspase-3 (B).
(D and E) *dronc* mutant wing discs at LW stage.
(D) Wg expression is normal (red) despite the presence of undead cells, confirmed by the presence of Hid protein (blue).
(E) *dronc* mutant wing discs contain no cleaved caspase-3.
(C, F–H) Diap1 degradation is not necessary for undead-induced proliferation.
(C) Expression of Diap1 protein in a control wing disc.
(F) Expression of Hid + P35 induces loss of Diap1 protein (red) in many posterior cells (green). Inset shows single-channel image of Diap1 expression.
(G–G') Expression of Dronc + P35 induces stronger overproliferation than Hid + P35, but Diap1 protein persists in most undead cells (G'). The A/P boundary is marked with a dotted yellow line.
(H) In *dp53* mutant discs, expression of Dronc + P35 has no effect on Diap1 expression (blue), and disc growth and morphology is normal. Dronc protein is shown in red.
(I–K) *dronc* is required for expression of *dp53* in undead cells.
(I and J) RNA in situ hybridization to detect *dp53* mRNA in wing discs expressing Hid + P35 from *dp53* mutant (I) and *dronc* mutant (J) larvae, showing that *dp53* mRNA is not induced in either mutant.
(K) *dp53* mRNA is strongly induced in wing discs expressing Dronc + P35. Bars indicate relative scale.

Blastema Formation during Leg Disc Regeneration Requires *dronc* and *dp53*

The parallels between the disc response to undead cells and the regeneration response of imaginal discs are striking, in that both induce growth arrest, changes in

the expression of pattern regulating genes, and compensatory proliferation [12, 14, 17]. The reduced survival of irradiated *dp53* mutants, combined with our observations that *dp53* mutants containing undead cells were unable to induce compensatory proliferation, thus suggested the possibility that *dp53* has a general role in allowing regeneration of damaged tissue. During imaginal disc regeneration, a proliferating blastema forms that supplies cells that will regenerate missing structures [10–12]. We tested the role of *dp53* in blastema formation by inducing a regeneration response in leg discs via an established method whereby ectopic expression of Wg in the “weak point”—dorsal proximal cells—of the prothoracic leg disc induces a proliferating blastema and leads to a regenerative response in a process virtually identical to that after disc fragmentation [17, 37]. We monitored blastema formation by labeling wild-type, *dp53* mutant, and *dronc* mutant prothoracic leg disc cells with BrdU (Figures 5I–5K). After expression of Wg for 40 hr, 59% of wild-type discs formed a blastema, consistent with previous reports [17]. In contrast, only 22% of *dp53* mutant and 15% of *dronc* mutant leg discs formed a blastema (Figure 5K). These data suggest that in addition to their role in promoting compensatory proliferation in response to undead cells, *dp53* and *dronc* contribute to blastema formation during a regeneration response. Collectively, these results support the idea that in the absence of *dronc* and *dp53*, extensive tissue damage leads to inefficient tissue repair.

Discussion

The repair of tissue after cellular damage can be critical to the survival of the animal. Previous studies demonstrated that undead cells stimulate the proliferation of neighboring cells, providing a model for how damaged and dying cells contribute to the replacement of lost tissue.

With this model, we find that the wing imaginal disc responds to this damage as a whole by deploying a multi-step process that ends with compensatory growth. We find that *dp53*, the sole p53 family member in *Drosophila*, functions in a *dronc*-dependent manner at each step of the tissue-replacement process. Furthermore, our experiments suggest that *dp53* and the initiator caspase *dronc* may be generally required for tissue recovery in imaginal discs, because we found that blastema formation was significantly impaired during regeneration induced in either *dp53* or *dronc* mutant leg discs.

dp53 Is Induced Independently of DNA Damage, but Requires Dronc Activity

Our data suggest that *dp53* is induced and becomes functional in undead cells by a mechanism that does not require DNA-damage sensing or activation of the stress kinase AMPK. Rather, we find that Dronc, an initiator caspase homologous to caspase-9, is necessary and sufficient to induce all aspects of the growth regulation by *dp53*. We do not know how Dronc activity results in *dp53* expression and activity in these cells, but many caspase substrates are not directly involved in apoptosis. As an example, one of the first substrates identified was the cytokine IL-1 β , which regulates many aspects of the inflammatory response [38]. We found that induction

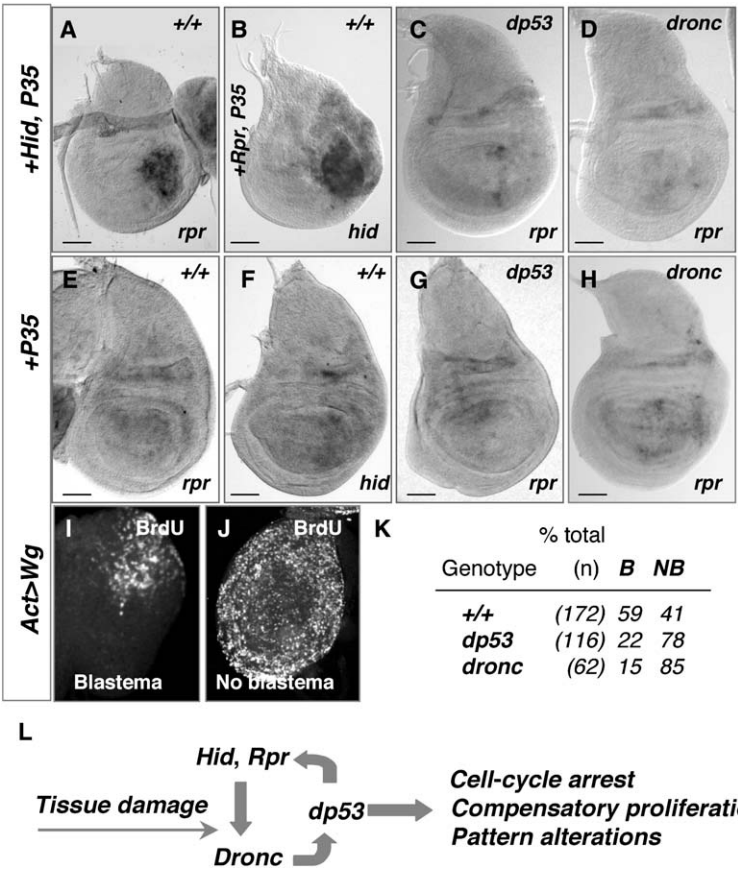


Figure 5. *dp53*, *Dronc*, and the Endogenous *hid* and *rpr* Loci Form a Regulatory Loop that Promotes Compensatory Proliferation

(A) 96 hr wing disc expressing *Hid* + *P35* in posterior cells. *rpr* mRNA is highly induced in the UAS-*Hid*-expressing undead cells.

(B) 96 hr wing disc expressing *Rpr* + *P35* in posterior cells. The undead cells express high levels of *hid* mRNA, from the endogenous *hid* locus.

(C and D) *dp53* and *Dronc* are required for expression from the *rpr* and *hid* loci in wing discs with undead cells.

(C) *dp53* mutant wing disc expressing *Hid* + *P35*. Loss of *dp53* prevents the expression of *rpr* mRNA.

(D) *rpr* expression is not induced by *Hid* + *P35* in *dronc*¹²⁹ mutant wing discs.

(E–H) *rpr* and *hid* mRNA expression in control discs expressing *P35* alone.

(E and F) Wild-type wing imaginal discs.

(G) *dp53* mutant wing disc.

(H) *dronc*¹²⁹ mutant wing disc. Bars in (A)–(H) indicate relative scale.

(I–K) Regeneration induced by ectopic expression of *Wg* requires *dp53* and *dronc* for blastema formation.

(I) *BrdU* labels only the dorsal proximal cells of the “weak point” in regenerating prothoracic leg discs.

(J) Prothoracic leg disc with no blastema.

(K) Percent of prothoracic leg discs that form a blastema after ectopic *Wg* expression in wild-type (+/+), *dp53*, or *dronc* mutant animals. See text for details. B, blastema; NB, no blastema.

(L) Model of *dp53* activation and its regulation of the processes associated with compensatory proliferation.

tory proliferation. Tissue damage due to undead cells results in the activation of *Dronc* (possibly in a nonapoptotic role, see [5]), and results in a dramatic increase in expression of *dp53*. How *dp53* expression is upregulated is unknown. The activity of *dp53* causes G2 arrest, compensatory proliferation, and also ectopic expression of *Wg* and *Dpp*. The induction of *hid* and *rpr* expression by *dp53* amplifies and sustains the process. All arrows indicate genetic relationships.

of *dp53* mRNA in undead cells is prevented in *dronc* mutant discs, and thus it is possible that a regulator of *dp53* is cleaved by *Dronc*, leading to its expression and ultimately to its ability to regulate the compensatory growth response in the imaginal discs. Regardless of the molecular mechanism, our data argue for direct communication between *Dronc* and *dp53* in response to tissue damage.

dp53: A Damage Monitor that Induces Compensatory Growth

Collectively, our experiments imply that *dp53* serves as a master coordinator of tissue repair in imaginal discs, regulating both cell-autonomous and non-cell-autonomous cell-cycle arrests, the expression of the pattern-regulating genes *wg* and *dpp*, and compensatory cell proliferation and growth. Based on our results, we suggest that cellular damage activates *Dronc*, which in a nonapoptotic role causes the induction of *dp53* mRNA and leads to *dp53* activity. We propose that *dp53* then acts as an overall damage monitor, in a role that includes its conserved functions in apoptosis (here, induction of *hid* and *rpr* expression) and growth arrest (by repression of *stg/cdc25*), but also allows for induction of signals that promote compensatory growth of the disc. Our results suggest that *dp53* monitors tissue damage

through a feed-forward loop with *Dronc* and the proapoptotic genes *hid* and *rpr*, which both amplifies and sustains the growth-regulating signal (Figure 5L).

An intriguing puzzle left unanswered by our results is why the growth response to undead cells occurs only several days after they are generated: both *HhGal4* and *EnGal4* drive expression of *Hid* or *Rpr* from early embryonic stages, yet even with careful observation we detected no growth phenotype until the middle part of the third instar. Caspases are active in cells expressing *Hid* or *Rpr* + *P35* at early time points, indicating that these cells are not immune to the apoptotic response early in development (data now shown). The genes involved in the apoptotic response are subject to many levels of control, including that by micro-RNAs (miRNAs). *Hid* protein expression, for example, is suppressed by *Bantam*, a miRNA highly expressed early in imaginal disc development, but declining as development progresses [39]. It is likely that *rpr* is also regulated by miRNA gene silencing [40]. Hence, the delay of the growth response in discs with undead cells may reflect a requirement for threshold levels of these factors to fully activate the feedback loop. At the very least it emphasizes that the regulation of growth and cell death during wing disc development is complex and has multiple inputs, many of which we have little knowledge.

Activity thresholds appear to play an important role in the processes induced by undead cells. We found that Dronc, for instance, is haploinsufficient for its effect in compensatory proliferation. It is possible that the apoptotic functions of Dronc require a relatively low activity level, but that high Dronc activity allows activation of the dp53-dependent tissue-damage response. Regulation of Dronc by critical activity thresholds could provide the animal some regenerative capacity and increase its chances for survival when conditions are appropriate for tissue repair.

dp53 Regulates *string/cdc25* to Activate a G2 Checkpoint

As expected given its role in coordinating many cellular behaviors, p53 modulates the activity or expression of myriad effectors [1]. Regulatory effectors of *Drosophila* p53 are only beginning to be identified, and our data add *stg/cdc25* to the list. In our experiments, one of the first detectable disc responses to undead cells is G2 arrest, mediated by loss of *stg* mRNA. Cdc25 is also regulated by vertebrate p53 but is inhibited post-transcriptionally by p53-dependent 14-3-3 activity [1]. Experiments with irradiated *dp53* mutant animals have not revealed a cell-cycle arrest role previously [19, 20, 23]. However, recent work indicates that dp53 also regulates a G1 checkpoint under conditions of metabolic stress [34]; thus, like vertebrate p53, dp53 can activate both a G1 and a G2 checkpoint in response to tissue stress. Other effectors and targets involved in the compensatory proliferation process remain unknown, although expression profiling experiments from irradiated *dp53* mutants identified several potential targets, several of which do not have obvious roles in cell death or DNA repair [19].

dp53 Coordinates Signaling Processes Leading to Damage-Induced Growth

How does dp53 control the signaling that leads to compensatory proliferation? The events that we observe—G2 arrests in two different cell populations, ectopic expression of *wg*, and compensatory growth—are all regulated by dp53. It is possible that dp53 directly and coordinately controls each of these processes by regulating the expression of specific effectors. However, because the response is both cell autonomous and non-cell autonomous, we favor the idea that these processes are interdependent, but sequentially activated. We envision that as a result of Dronc activation in undead cells, dp53 induces loss of *stg*, leading to G2 arrest, and *hid* and *rpr* expression, initiating the feedback loop. We postulate that cells then synthesize factors that stimulate their survival and proliferation. The non-cell-autonomous arrest in the anterior compartment may be a secondary effect of undead cells in the posterior. We have observed high levels of TUNEL activity in the anterior cells of these discs (data not shown), which could feasibly activate dp53 in those cells. However, we did not detect *dp53* mRNA in anterior cells. One possibility is that the DNA fragmentation resulting from dying anterior cells could activate ATM and Chk2 in those cells. Consistent with this, although loss of either of these kinases did not affect undead cell induction of *Wg* expression or compensatory growth, the cell-cycle arrest in anterior

cells was reduced in a fraction of *atm* and *chk2* mutants (Figure S2 and data not shown).

What is the growth-stimulating signal induced by undead cells? While its identity is still unclear, both *Wg* and *Dpp* have been implicated in this role [7, 13]. This makes sense, because *Wg* and *Dpp* are the major pattern organizers of all imaginal discs and are also involved in regulating their growth, and furthermore they are known to be induced in disc regeneration [14, 15, 41]. However, although *wg* and *dpp* are ectopically expressed in undead cells, we find that targets of both are sharply downregulated, specifically in the undead cells (Figure S4). Our data also show that undead cells are able to proliferate and contribute to the compensatory growth. Thus, although the nonautonomous stimulation of growth (anterior cells near the A/P boundary) could be due to increased *Dpp* signaling, we suspect that the autonomous growth stimulation is due to other, unidentified factors.

Evolutionary Considerations

We have identified a growth-regulatory role for dp53 that seems counter to its role as a tumor suppressor in vertebrates. However, we speculate that the ability of dp53 to sense and respond to tissue damage and promote compensatory proliferation and regeneration in *Drosophila* reflects an ancestral function, aspects of which have been appropriated for developmental processes and distributed among p53, p63, and p73 during vertebrate evolution. Although p63 and p73 initially were proposed to have evolved as duplications of p53, reanalysis of the phylogenetic relationship between the three family members has suggested that p63 may be the ancestral gene [26, 42, 43]. p63 and p73 are structurally similar to p53 but contain an additional SAM domain. *dp53* is the sole member of the family encoded in the *Drosophila* genome, and although dp53 does not contain a SAM domain, based on the sequence of the DNA binding domain, the most highly conserved region of p53, it is more related to vertebrate p63 than to p53 [26]. After irradiation, cell-cycle arrest is not p53 dependent in either *Drosophila* or the nematode *C. elegans*, and therefore it has been proposed that the ancestral p53 function is apoptosis, rather than a “repair, then death” response when damage cannot be repaired [20]. Our experiments argue that as in vertebrates, *dp53* plays a role in cell-cycle arrest after tissue damage. The additional functions of dp53 in promoting cell proliferation may have been conserved in p63, which regulates progenitor cell renewal in the epidermis. Other processes that require cell renewal may also be regulated by dp53. For example, *dp53* mutants are reported to have fertility defects [20, 44], so it is tempting to speculate that stem cell renewal in the gonad requires this previously unappreciated role of dp53.

Conclusions

How cell death and cell proliferation are coordinated during development and after stress is a fundamental question that is key to our understanding of growth regulation. We have found that dp53 is required for the compensatory growth induced after tissue damage, which could reflect an ancestral function of p53 in tissue repair and cell renewal.

Experimental Procedures

Fly Strains

The following strains were used: UAS-P35 [45]; UAS-Hid [5]; UAS-Rpr (gift of J. Abrams), Act>Draf>lacZ [27]; *dp53^{ns}* [20]; *dp53^{5a-1-4}* [21]; *atm⁶*, *atm³* [33]; *chk2^{P6}* (also called *mnk^{P6}*) [19]; EnGal4, UAS-GFP [28]; HhGal4, UAS-GFP; *dronc¹²⁹*/TM6B [36]; *Df(3L)XR38*/TM6B; *hid^{A22}*/TM6B; *Df(3L)H99*/TM6B; *hid^{P05014}*/TM6B; *hid^{X14}*/TM6B; UAS-Dronc⁸⁰ [5]; UAS-Wg; Act>y+>Gal4, UAS-GFP; Dpp-lacZ; Brk-Z, VgBE-Z; VgQE-lacZ. Unless otherwise indicated, fly strains are described on <http://flybase.bio.indiana.edu>.

Fly Husbandry

Eggs from appropriate crosses were collected on yeasted grape plates for short periods (2–3 hr). After hatching, larvae were transferred to standard molasses food vials (≤ 50 /vial) supplemented with fresh yeast and raised at 25°C for defined periods of time, as described [28, 29].

Cell-Proliferation Rate Measurements

The Flp-out Lac-Z cassette [27] was used to generate neutral clones. Larval heat shocks to activate Flp recombinase expression were performed at 37°C for 20 min at 70 hr AEL and allowed to grow until 96 hr AEL. The location of each clone in the wing disc was recorded as medial or lateral (with respect to the A/P boundary) in the anterior or posterior. Cell doubling times were determined by counting the number of cells in each clone with the formula $\log 2$ (hr)/log N, where N = median cell number/clone and hr = age of the clone [28].

Flow Cytometry

Wing discs were dissected at the time points indicated and dissociated into single-cell suspensions in Trypsin-EDTA supplemented with Hoechst 33342 [28]. Cell cycles were analyzed as described previously [28], with a Becton Dickinson LSR II with FACS Diva software.

Leg Disc Regeneration

Regeneration was induced in leg imaginal discs as described [17]. In brief, larvae of the genotype *yw*hslp; Act>y+>Gal4, UAS-GFP/UAS-Wingless; *dp53^{ns}* (or *dronc¹²⁹*) were subjected to a 2 hr heat shock at 37°C at 72 hr AEL. This induced Wingless in >95% of cells and led to regeneration in the first leg discs. After 40 hr, blastema formation was monitored by BrdU labeling of discs for 10 min, and the percent of discs with a regenerating blastema was scored as described [17, 37].

Immunocytochemistry

Fixation and immunocytochemistry of imaginal discs were carried out as described [31]. RNA in situ hybridizations were carried out with digoxigenin-labeled RNA probes [31]. TUNEL assays were carried out with Apoptag Red (Intergen); a detailed protocol is available upon request. BrdU assays were carried out as described [29] with modifications (protocol available upon request). Images were acquired with Apotome software and a Zeiss Axioplan 2 microscope with an Orca-100 CCD camera (Hamamatsu) and processed with Photoshop (Adobe) software. The following antibodies and dilutions were used: rabbit anti-p-Smad, 1:4000 (gift of E. Laufer); rabbit anti-Vg, 1:100 (gift of S. Carroll); mouse anti-Cut, 1:50 (DSHB); mouse anti-Wg, 1:30 (DSHB); rabbit anti- β -gal, 1:2000 (Cappel); mouse anti-Diap1, 1:200, and rabbit anti-Hid, 1:1000 (gifts of B. Hay); rabbit anti-Diap, 1:100 (gift of H. Steller); rabbit anti-cleaved Caspase 3, 1:100 (Cell Signaling); mouse anti-Digoxigenin, 1:2000 (Roche); mouse anti-BrdU, 1:100 (Roche); rabbit anti-p-AMPK, 1:250 (Cell Signaling); guinea pig anti-Dronc, (1:1000) [36].

Supplemental Data

Four Supplemental Figures can be found with this article online at <http://www.current-biology.com/cgi/content/full/16/16/1606/DC1/>.

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